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Fast LC-MS quantification of ptesculentoside, caudatoside, ptaquiloside and corresponding pterosins in bracken ferns

Vaidotas Kisielius[a,b,*], Dan Nybro Lindqvist[a], Mikkel Boas Thygesen[c], Michael Rodamer[d], Hans Christian Bruun Hansen[b], Lars Holm Rasmussen[a]

[a] Department of Technology, University College Copenhagen, Sigurdsgade 26, 2200 Copenhagen, Denmark
[b] Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark
[c] Department of Chemistry, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen, Denmark
[d] Agilent Technologies, Hewlett-Packard St. 8, 76337 Waldbronn, Germany

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ABSTRACT

Ptaquiloside (PTA) is an illudane glycoside partly responsible for the carcinogenicity of bracken ferns (*Pteridium* sp.). The PTA analogues ptesculentoside (PTE) and caudatoside (CAU) have similar biochemical reactivity. However, both compounds are highly under-investigated due to the lack of analytical standards and appropriate methods. This study presents a robust method for preparation of analytical standards of PTE, CAU, PTA, the corresponding hydrolysis products: pterosins G, A and B, and an LC-MS based method for simultaneous quantification of the six compounds in bracken. The chromatographic separation of analytes takes 5 min. The observed linear range of quantification was 20–500 μg/L for PTA and pterosin B, and 10–250 μg/L for the remaining compounds ($r > 0.999$). The limits of detection were 0.08–0.26 μg/L for PTE, CAU and PTA and 0.01–0.03 μg/L for the pterosins, equivalent to 2.0–6.5 μg/g and 0.25–0.75 μg/g in dry weight, respectively. The method was applied on 18 samples of dried fern leaves from 6 continents. Results demonstrated high variation in concentrations of PTE, CAU and PTA with levels prior to hydrolysis up to 3,900, 2,200 and 2,100 μg/g respectively. This is the first analytical method for simultaneous and direct measurement of all six compounds. Its application demonstrated that bracken ferns contain significant amounts of PTE and CAU relative to PTA.

1. Introduction

Bracken is a group of carcinogenic ferns comprising the genus *Pteridium* (Pteridaceae) [1]. *Pteridium* is found at all continents except Antarctica and is regarded as the fifth most abundant plant in the world [2–4]. Under favourable conditions bracken can proliferate at extreme rates. In the United Kingdom *Pteridium aquilinum* L. (Kuhn) covers 1.7 million ha which corresponds to 7.3% of the total land surface area of the country [5]. Increase in abundance and distribution of bracken is observed in many parts of the world and is predicted to continue due to changing climate, deforestation and other changes in land management [6,7].

Bracken is the only plant that is well-documented to naturally cause cancer in animals [8]. Several studies link bracken carcinogenicity to the illudane glycoside ptaquiloside (PTA) [9–12]. PTA is found in variable concentrations in the ferns reaching levels of 45,000 μg/g [13]. PTA is a pre-carcinogen, where the genotoxic effect is caused by a highly reactive conjugated dienone formed from PTA as result of hydrolysis [14,15]. The International Agency for Research on Cancer under WHO evaluates the evidence of bracken carcinogenicity in experimental animals as sufficient and classifies the plant as possibly carcinogenic to humans [16,17]. Nevertheless, traditional food containing bracken can be found in many nations of the world [18,19]. Correlation between human consumption of bracken and upper gastrointestinal tract cancers have been identified in Japan, Brazil and Venezuela [20–22].

PTA was identified in 1983 simultaneously by a Dutch and a Japanese research groups [23,24]. The structure was elucidated by ^{1}H NMR using PTA extracted from *Pteridium aquilinum* and *Pteridium aquilinum* subsp. *latiusculum* (Desvaux), respectively. Later on, several PTA-like compounds were isolated from other species of bracken: isoptaquiloside (PTA isomer) and caudatoside (CAU) from *Pteridium caudatum* (L. Maxon) [25], ptaquiloside Z from *Pteridium caudatum* [26] and hydroxyl-ptaquiloside ptesculentoside (PTE) from *Pteridium esculentum* (G. Forst.) [27] (concentrations unspecified). More PTA analogues are known in *Hypolepis punctata* and *Dennstaedtia hirsta*

* Corresponding author at: Department of Technology, University College Copenhagen, Sigurdsgade 26, 2200 Copenhagen, Denmark.
E-mail address: vaki@kp.dk (V. Kisielius).

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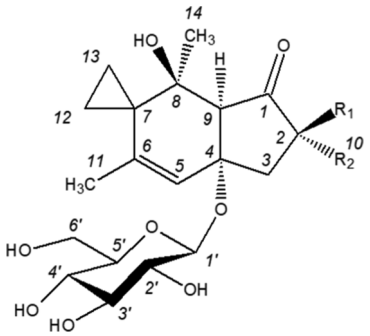
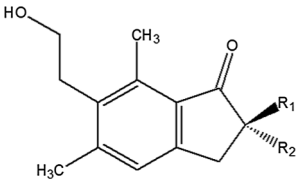
Table 1
Structures of analytes in decreasing order of polarity with numbering of carbon atoms of PTA. The cyclopropane functional group causing formation of dienone agents comprises atoms No. 7, 12 and 13.

Compound	PTE	CAU	PTA	Pterosin G	Pterosin A	Pterosin B
Common molecular structure						
R_1	H	CH_3	H	H	CH_3	H
R_2	CH_2OH	CH_2OH	CH_3	CH_2OH	CH_2OH	CH_3
Molecular Formula	$C_{20}H_{30}O_9$	$C_{21}H_{32}O_9$	$C_{20}H_{30}O_8$	$C_{14}H_{18}O_3$	$C_{15}H_{20}O_3$	$C_{14}H_{18}O_2$
CAS number	–	–	87625-62-5	35964-50-2	35910-16-8	34175-96-7
Estimated LogP (Source: PubChem)	−2	−1.4	−1.3	1.9	2.2	2.5

(Pteridaceae), like hypolosides A, B and C [28]. However, these have never been reported in bracken. PTE, CAU and PTA are believed to comprise the majority of illudane glycosides in bracken [29] (Table 1). The content of PTA is known to be quite variable due to environmental and genetic factors. Large temporal and spatial variation is encountered and PTA hot-spots are found in some geographical regions. The hot-spots are correlated with bracken induced chronic diseases among farm animals. Similarly, proximity to bracken is a potential risk factor for human gastric and oesophageal cancers [30–34].

Quantification of PTA in larger surveys is based on aqueous extraction of PTA from dried bracken. However, sample preparation and extraction can cause transformation of PTA into pterosin B as result of hydrolysis under alkaline or acid conditions, exposure to heat and/or enzymes [35]. As a result, PTA quantification methods comprise either individual measurements of PTA and pterosin B (direct method) [36–38] or measurement of pterosin B after deliberate total conversion of PTA (conversion method) [39–43]. Similar conditions are expected for PTE and CAU requiring quantification of equivalent pterosins G and A (Table 1). The only way to avoid formation of pterosins is to use hot water or hot methanol extraction of freshly harvested plant material [35]. This is however inapplicable for high-throughput screening surveys.

The analytical methods for quantification of PTA and associated pterosin B are based on HPLC-UVVIS, LC-MS, LC-MS/MS and GC-MS. The LOD range from 0.014 to 0.6 μg/L for PTA and 0.008 to 500 μg/L for pterosin B. A single GC-MS based method exists for the simultaneous quantification of PTE, CAU and PTA (LOD not specified) [29]. It is based on the conversion of the illudane glycosides into pterosins as the former cannot be analysed by GC. The disadvantage of the conversion approach is that it does not differentiate the pterosins originally present in analytical samples from the pterosins intentionally generated in sample treatment. The issue can be handled by sequential analysis of sample fractions containing pterosins and fractions containing illudane glycosides converted into pterosins. However, this is time-consuming and a direct method is desired.

Bracken carcinogenicity is amplified by the number of PTA analogues potentially being present in the ferns [44]. Most studies evaluating bracken toxicity do not quantify or discuss PTA analogues. Hence, the purpose of this study was to develop a fast, direct and versatile method for high-throughput screening of bracken for content of PTE, CAU, PTA and pterosins G, A and B. In addition, a novel method for purification of the analytes is presented.

2. Materials and methods

2.1. Solvents and chemicals

LC-MS grade acetonitrile was obtained from Merck Millipore (LiChrosolv hypergrade for LC-MS, Germany). LC-MS grade methanol was obtained from Honeywell (LC-MS Chromasolv, Germany). Water for preparative HPLC and LC-MS (electrical resistivity 18.2 MΩcm, TOC less than 2 μg/L) was dispensed from Satorius Arium Pro Ultrapure water system (Germany). Acids and bases (sodium hydroxide, trifluoroacetic, formic and hydrochloric acids) were all of analytical grade from Sigma-Aldrich (Denmark). Polyamide was obtained from Fluka Analytical Sigma-Aldrich co (Polyamide for column chromatography 6, Germany).

2.2. Analytical instruments

For preparation of analytical standards Shimadzu LC 20-AD semi-preparative LC system equipped with Shimadzu SPD-10A UV–VIS detector and Hitachi D 2000 Chromato-Integrator were used. ^{1}H NMR spectra were recorded with Bruker Avance 300 MHz NMR spectrometer. The method of chromatographic separation and quantification of analytes was developed in an analytical Agilent 1260 Infinity HPLC system equipped with an Agilent 6130 Single Quadrupole mass spectrometer. UV absorption profiles of the analytes were obtained with an Agilent 1260 DAD VL detector.

2.3. Collection of bracken samples

The method was applied on 18 samples of dried grinded fern leafs. The species and geographic locations are provided in Table 9. The collection time, sampling, preservation and storage methods applied are provided in Table S1 of Supplementary Material (SM).

2.4. Preparation of analytical standards

No certified reference materials of PTE, CAU and PTA exist and therefore analytical standards have to be prepared from the compounds extracted, isolated and purified from bracken. For preparation of analytical standards, a record of bracken containing sufficient amounts and proportion of PTE, CAU and PTA to be extracted was found in the literature (*Pteridium esculentum* in Bribie Island, South East Queensland, Australia [29]). Recently emerged furled bracken fronds were collected

Table 2
Observed UV absorption profiles ($\lambda_{max1} > \lambda_{max2} > \lambda_{max3}$) and literature-based molar extinction coefficients of pterosins at 217 nm.

	λ_{max1} (nm)	λ_{max2} (nm)	λ_{max3} (nm)	ε at λ_{max1} (abs/mol/L) [29,47]
PTE	202	none	none	n/a
CAU	202	none	none	n/a
PTA	202	none	none	n/a
Pterosin G	217	263	306	26,915
Pterosin A	217	263	306	34,674
Pterosin B	217	260	304	37,154

in the reported location in 2017, freeze-dried, grinded to fine powder and kindly provided by Prof Mary T. Fletcher (The University of Queensland, Australia). For isolation of the compounds 20 g of the powder were mixed with 200 mL deionized water in a glass bottle, shaken at 100 rpm for 30 min. on a shaking table and centrifuged for 20 min. at 2500 g at 1 °C (Sigma Zentrifugen 4 K15). The supernatant was filtered through a 2 μm pore size filter paper (Whatman No. 41, Sigma-Aldrich, Germany) under vacuum. Two cylindrical glass funnels (Ø = 1.5 cm; l = 20 cm, BIO-RAD, Econo-Column, USA) were dry packed with approximately 10 g of polyamide. The aqueous extract was passed through the columns at an approximate speed of 3 mL/min to retain hydrophobic impurities and pterosins [45]. Extra 20 mL of deionized water was added to each column to fully elute the illudane glycosides. The bottles and flasks used throughout the process were wrapped in aluminium foil and kept in ice bath to prevent photolysis and thermal degradation.

The analytes were retrieved from the eluate by solid phase extraction. OASIS HLB 6 cc 200 mg cartridges (Waters co, Ireland) were conditioned with 12 mL of deionized water, loaded with 30 mL of the eluate, washed with 5 mL of deionized water and eluted with 5 mL of 40% methanol at an approximate speed of 3 mL/min. The pre-concentrated eluate was further purified by semi-preparative HPLC system using a reversed-phase Agilent ZORBAX Eclipse XDB-C18 5 μm 9.4 × 250 mm column (C18 5 μm 9.4 × 15 mm guard column) at ambient temperature (injection volume 5 mL, mobile phase 40% methanol, flowrate 4 mL/min, λ 220 nm). The retention times of the compounds at 23 °C were 7.7 min for PTE, 14.5 min for CAU and 19.9 min for PTA (Fig. S1 of SM). The injection was repeated 8 times to accumulate approx. 50–80 mL of each fraction. The compound identities were confirmed in LC-MS and the methanol was removed from the eluates using a rotary evaporator (BUCHI Rotavapor R-210) at 35 °C water bath temperature.

Attempts to isolate PTE, CAU and PTA demonstrated that pure crystals of the glycosides instantly absorb ambient humidity and the crystals transform into sticky transparent gels. Concentration of the dried least hydroscopic glycoside PTA was determined as the reference to calculate concentrations of the rest of the analytes. The aqueous solution of PTA was frozen to −80 °C and freeze-dried at −55 °C (freeze-drier: Scanvac Coolsafe 55-4) in a 30 mL amber bottle covered with perforated aluminium foil. After approximately 36 h the pressure in the freeze-dryer vacuum compartment was gradually increased supplying dried nitrogen gas (nitrogen generator: Peak Scientific NM32LA). The bottle with dry PTA was immediately closed and transported for weighing and handling in a glove bag (Atmosbag 39″ × 48″, Sigma-Aldrich, Germany) inflated with dried nitrogen gas and loaded with silica gel beads to absorb humidity. Several minutes after a humidity meter in the glove bag showed relative humidity lower than lowest detectable (20%), the NMR tube was filled with 2.9 mg PTA, 950 μL D_2O (99.9 atom % D, Sigma-Aldrich, Germany), 25 μL 20 mM KH_2PO_4 and 25 μL 20 mM 3-(trimethylsilyl propionic-2,2,3,3-d4 acid, sodium salt solution (internal standard) (99.9% pure; ≥98.0 atom % D, Sigma-Aldrich, Germany). The data of NMR analysis processed with Mestrelab Research S.L. analytical chemistry software *MestReNova v12.0.3* resulted in a purity of PTA of 94% based on integration of the H atom (H-5) attached to C-5 (5.80 ppm) and 90% based on the H atom (H-14) attached to C-14 (1.32 ppm) (Table 1) relative to the internal standard. ^{1}H NMR (D_2O, 20 mM KH_2PO_4, 300 MHz): δ 5.80 (s, 1H; H-5), 4.77 (overlapped by HDO; H-1′), 3.97 and 3.77 (2dd, J = 12.5 and 2.0 Hz and J = 12.5 Hz and 5.3 Hz respectively, 2H; H-6′), 3.56–3.41 (m, 3H; H-3′ and H-4′ and H-5′), 3.32 (dd, J = 9.0 and 8.0 Hz, 1H; H-2′), 2.78 (d, J = 1.4 Hz, 1H; H-9), 2.57–2.44 and 2.05–1.97 (2 m, 3H; H-2 and H-3), 1.58 (d, J = 1.3 Hz, 3H; H-11), 1.32 (s, 3H; H-14), 1.13 (d, J = 6.7 Hz, 3H; H-10) , 1.01–0.87 and 0.79–0.64 (2 m, 4H; H-12 and H-13) (Fig. S2 of SM). For representation of the purity of PTA the average (92%) was used.

Half of the volume of obtained aqueous solutions of PTE and CAU were allocated for production of pterosins G and A. An aqueous solution of known concentration of PTA was prepared for production of pterosin B. The glycosides were converted by mixing the aqueous solutions with 75 μL of 1 M NaOH per 1 mL of solution, submerging for 15 min. in 35 °C water bath, allowing to cool to room temperature and mixing with 75 μL of 2.5 M trifluoroacetic acid per 1 mL of solution [46]. The 100% conversion rates were confirmed in LC-MS by observed disappearance of the glycosides and occurrence of pure pterosins. The concentration of the obtained solution of pterosin B was determined applying 1:1 M conversion ratio from the original solution of PTA. The concentrations of the obtained solutions of pterosins G and A were determined comparing absorption of pterosins G, A and B in 217 nm UV with molar extinction coefficients ε (Table 2). The concentrations of solutions of PTE and CAU were back-calculated applying 1:1 M conversion ratios of these compounds to pterosins G and A (Fig. 1). In order to prevent hydrolysis and degradation of the obtained analytical standards, the aliquots were preserved in 50% v/v MeOH and stored at −80 °C.

2.5. LC-MS method development

2.5.1. Chromatographic separation

A high-throughput reversed-phase column was selected for fast analysis of the hydrophilic compounds (Agilent InfinityLab Poroshell 120, 2.7 μm EC-C18, 3.0 × 50 mm column; 2.7 μm EC-C18 3.0 × 5 mm guard column). The chromatographic separation was developed towards full resolution of all analytes and fast elution of the latest eluting analyte in different gradient steepness of methanol and acetonitrile. The peak resolution values were monitored in Agilent OpenLab CDS ChemStation C.01.07.

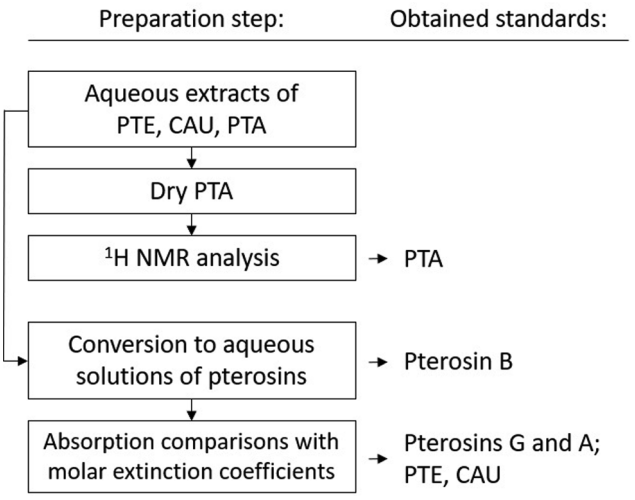


Fig. 1. Flowchart of preparation of analytical standards.

Table 3
Mass fragments in chronological order of retention times. The masses monitored in SIM are underlined.

Peak No.	Compound	Monoisotopic mass (g/mol)	*m/z* 200–460 (relative abundance ≥ 10%, excluding isotopes)
1	PTE	414.2	217.1 [M-glucose-H_2O + H]$^+$, <u>235.1</u> [M-glucose + H]$^+$, 437.2 [M + Na]$^+$, 453.1 [M + K]$^+$
2	CAU	428.2	231.1 [M-glucose-H_2O + H]$^+$, <u>249.1</u> [M-glucose + H]$^+$, 451.2 [M + Na]$^+$
3	pterosin G	234.1	<u>235.1</u> [M + H]$^+$, 257.1 [M + Na]$^+$
4	PTA	398.2	201.1 [M-glucose-H_2O + H]$^+$, <u>219.1</u> [M-glucose + H]$^+$, 421.2 [M + Na]$^+$, 437.1 [M + K]$^+$
5	pterosin A	248.1	<u>249.1</u> [M + H]$^+$, 271.1 [M + Na]$^+$
7	pterosin B	218.1	<u>219.1</u> [M + H]$^+$

Table 4
Fractional factorial experimental design.

Parameter		Factor −					Factor +		Method of Rai et al. [30] for PTA and pterosin B				
A: Extraction duration (min.)		20					70		60				
B: Solvent composition		100% water					20% v/v MeOH		100% water				
C: pH (buffer) of extraction		4.5 (0.1 M NH_4AC)					6 (0.1 M NH_4AC)		Approx. 5 (not buffered)				
D: Sample/solvent		40 mg/40 mL					120 mg/40 mL		100 mg/40 mL				
Method		1	2	3	4	5	6	7	8	9	10	11	12
Parameter	A	+	+	−	+	+	+	−	−	−	+	−	−
	B	−	+	+	−	+	+	+	−	−	−	+	−
	C	−	−	+	+	−	+	+	+	−	−	−	−
	D	+	−	−	+	+	−	+	+	+	−	−	−

2.5.2. Optimisation of mass detector

The eluents were selected comparing the strengths of the signals of analytes forming with adducts of H^+, Na^+, K^+, Li^+ and NH_4^+ in different combinations of LC mobile phase compositions. Electrospray ionisation (ESI) parameters were optimised in full factorial experimental design wherein the effects on signal strengths by 4 factors at 3 levels were compared in 81 (3^4) possible combinations (cone voltage: 1,000–3,000–5,000 V; drying gas flow: 7–10–13 L/min; drying gas temperature: 150–250–350 °C; nebulizer pressure: 20–40–60 psi). The ions underlined in Table 3 were monitored in the mass spectrometer operated in single ion mode (SIM).

2.6. Validation of the analytical method

To delineate the linearity range, 3 identical sets of 7 vials with different concentrations for each analytical standard were independently made (in total 21 vials for each compound) and analysed in a randomised order. The average SIM areas measured from the 3 vials containing the same concentrations of a given analyte were used for construction of the calibration curves. The linearities of PTE, CAU, pterosins G and A were explored in the 10–250 µg/L range, whereas the linearities of PTA and pterosin B were explored in the 20–500 µg/L range. LOD was calculated as 3.3 times the $SD_{intercept}$/slope of the calibration curves. LOQ was calculated as 10 times the $SD_{intercept}$/slope of the calibration curves. Precision of the instrument was calculated using relative standard deviation (RSD) for replicate injections (n = 7) of analytical standards injected from the same vial. Precision of the standards was calculated using RSD for single injections of 3 different independently made vials of identical concentrations (n = 3). The precision of instrument and standards were expressed as the range from the lowest to the highest RSD observed.

2.7. Robustness of analytical standards during storage

Due to instability of the illudane glycosides [35] robustness of the analytical standards during storage were tested. The identical triplicate sets of standards used for making the calibration curves were distributed to store one set of each compound at −20 °C, +5 °C and approx. +23 °C (room temperature). The slopes, linearities and pairwise t-tests (95% confidence interval) of the calibration curves produced from the samples stored for 3 and 12 weeks were compared.

2.8. Extraction of illudane glycosides and pterosins from bracken

2.8.1. Extraction method development

Sample preparation was optimised based on a method for extraction of PTA and pterosin B from dry bracken powder by Rai et al. [30]. The extracting solvent was made adjusting 0.1 M ammonium acetate buffer with HCl to pH 4.5 or 6.0 (pH Meter: MeterLab PHM220 Lab). The extraction was performed by mixing the dry plant powder with 40 mL of the extracting solvent in a Nunc 50 mL conical polypropylene centrifuge tubes (Thermo Fisher Scientific, Korea) for 20 min at 75 rpm (mixer: ELMI Intelli-Mixer RM-2L). The influence of 4 factors at 2 levels on the extraction efficiency of the 6 targeted compounds were compared in a fractional factorial experimental design (Table 4).

A method extracting the highest amount of each of the 6 targeted compounds per mass of the dry bracken powder was identified. Efficiency of the given method for the particular compound was equated to 100% (the maximum yield). The concentrations of each compounds extracted by the other methods were expressed as relative concentrations (x % of the maximum yield). The method yielding the highest average relative concentrations of all 6 compounds was selected as optimal.

2.8.2. Precision and accuracy of the extraction method

The precision of the extraction method was determined as RSD for the measured concentrations of all compounds obtained performing 7 parallel extractions from the same sample. The accuracy of the extraction method was tested performing parallel extractions by replacing the extracting solvent with 25%, 50% and 100% of the previously made extract of the same plant sample. The accuracy of the method was determined as the recovery percentage of all targeted compounds in the obtained more concentrated extraction solutions.

2.9. Matrix effects

The matrix effects of the plant extracts were tested injecting them into LC-MS as pure extracts and diluted 2, 5, 10, 15, 20, 35 and 50 times. Dilution was made with 50% v/v MeOH in a filtering vial (Syringeless filter device Mini-Uniprep, GE Healthcare Life Sciences,

UK). Suspended plant particles were removed by the 0.2 μm pore size membrane in the vial, and the measurements were performed injecting 20 μL of the solution into LC-MS directly from the vial. The curves of the measured concentrations versus the dilution factors were plotted and the ranges of linearity were explored. The matrix analyses were made on plant samples that naturally contained the 6 targeted compounds and represented different subspecies, origin and drying methods: freeze-dried *Pteridium esculentum* from Australia (sample No. 12) and plant-press dried *Pteridium aquilinum* from Sweden (sample No. 4).

2.10. Statistical analysis

Data analysis (linear regression, descriptive statistics, etc.) were performed with Microsoft Excel 2016. Effects of parameters applied optimising methods of analyte extraction and MS ionisation were examined with data analysis software Minitab 18 and graphical representation software Plotly.

3. Results

3.1. Preparation of analytical standards

Previously reported methods of PTA isolation depend on its sorption to XAD-2 polymeric absorbent [8,38,42,43,48,49]. The developed method compared with the most recent method of PTA isolation [38] showed that application of disposable OASIS HLB cartridges requires less labour and results in 19% loss of the mass of PTA on the absorbent in contrast to 72% loss in XAD-2.

3.2. LC-MS method validation

The analytes were separated in the analytical HPLC system thermostated at 35 °C at 1 mL/min flow with a mobile phase comprising water (eluent A) and acetonitrile (eluent B) both with 0.1% v/v formic acid in the following gradient elution: 0–1 min 10% B; 3 min 35% B; 4–4.5 min 95% B; 4.6–5 min 10% B. The chromatographic separation monitored with UV (injection volume 20 μL, λ 220 nm) is displayed in Fig. 2.

The chromatographic separation yielded full resolution of the least resolved peaks (No. 3 and 4). Nevertheless, in case of overlap the temperature of the column compartment could be increased to distribute resolution of the peaks No. 3–5 more evenly. It has been demonstrated that the increase of temperature to 60 °C has little or no effect on quantification of the analytes (Table S2 of SM).

Full factorial experimental design of ESI parameter analyses revealed the following set of parameters as the most optimal: cone voltage: 3,000 V; drying gas flow: 13 L/min; drying gas temperature: 350 °C; nebulizer pressure: 40 psi. (Effects of all parameters on ESI efficiency analysed and graphically presented in Figs. S4 and S5 of SM). The optimised ESI parameters yielded 2.2, 2.4 and 2.2 times higher responses of PTE, CAU and PTA respectively than application of the set of ESI parameters reported in PTA study with the same instrument by Rai et al. [30].

Table 5
Validation data of the LC-MS method. Compounds are listed in the descending order of the slopes of calibration curves.

Compound	Calibration range (μg/L)	LOD (μg/L)	LOQ (μg/L)	Precision of the instrument (%; n = 7)	Precision of standards (%; n = 3)
PtrA	10–250	0.02	0.05	1.16–1.81	1.27–5.34
PtrB	20–500	0.03	0.09	0.52–0.81	0.46–2.09
PtrG	10–250	0.01	0.03	0.36–2.70	1.46–3.26
PTA	20–500	0.22	0.68	1.07–3.16	0.09–7.24
PTE	10–250	0.08	0.25	1.38–5.79	3.32–7.22
CAU	10–250	0.26	0.78	3.08–4.59	4.68–8.61

The correlation coefficients (r) of the calibration curves gave excellent linearities with values > 0.999 for all 6 compounds over the concentration ranges investigated (10–250 or 20–500 μg/L) (Fig. S6 of SM). LOD, LOQ and the ranges of precision of instrument and standards are provided in Table 5.

3.3. Robustness of analytical standards during storage

The glycosides kept for 3 weeks at room temperature hydrolysed, calibration curves did not follow linear patterns and data are not shown. The parameters of calibration curves of analytical standards kept in −20 °C and + 5 °C for 3 and 12 weeks are provided in Tables 6 and 7.

The test demonstrated that analytical standards of the illudane glycosides can be kept in a fridge or a temperature-controlled autosampler (5 °C) for at least 3 weeks. However, after 12 weeks the calibration curves of the stored samples expressed poor linearity (≤0.991) and recovery in comparison to the curves stored for the same period in −20 °C (≤86.4). The analytical standards of pterosins could be stored at 5 °C for at least 12 weeks.

3.4. Sample preparation

Sample preparation method No. 3 providing the highest average extraction of compounds was identified as the optimal method

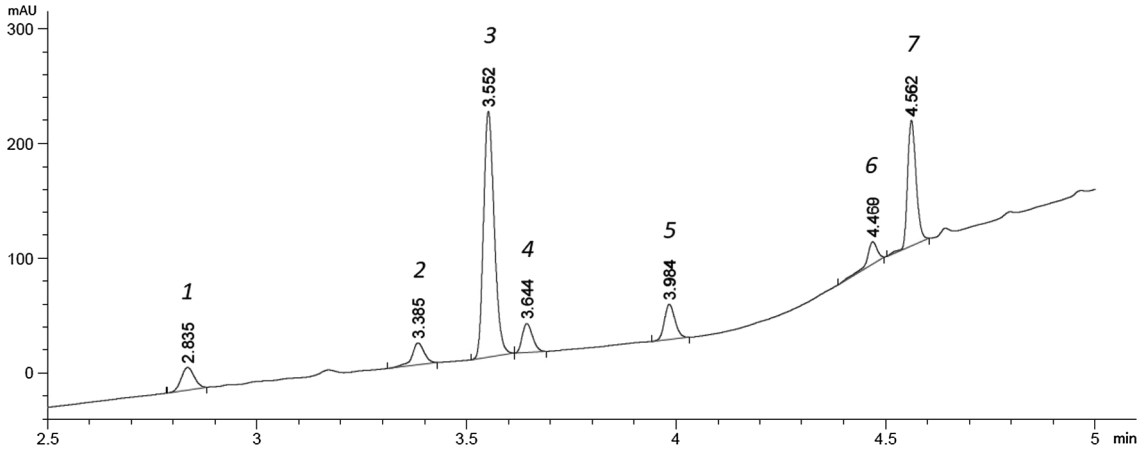


Fig. 2. Chromatogram of the compounds measured at 220 nm. 1: PTE (5,300 μg/L); 2: CAU (4,200 μg/L); 3: pterosin G (4,500 μg/L); 4: PTA (3,400 μg/L); 5: pterosin A (400 μg/L); 6: pterosin G methyl analogue (can form with methanol residues in incomplete conversion of PTE to pterosin G [50]); 7: pterosin B (1,300 μg/L).

Table 6
Comparison of calibration curves of analytical standards of illudane glycosides stored in different temperatures in time.

Compound	−20 °C			5 °C			Pairwise *t*-test
	Slope	Intercept	r	Slope	Intercept	r	Recovery %
3 weeks							
PTE	127	482	0.999	123	261	0.997	96.4 ± 3.79
CAU	63	605	0.993	60	350	0.992	96.9 ± 2.95
PTA	197	4,530	0.995	188	5,310	0.992	108 ± 12.6
12 weeks							
PTE	522	446	0.999	364	2,250	0.955	66.9 ± 4.22
CAU	309	212	0.999	204	1,280	0.937	71.9 ± 6.46
PTA	629	6,620	0.999	481	12,200	0.991	86.4 ± 6.6

(Table 8). No significant effect of a single parameter on the extraction efficiency was observed (Effects of all parameters on extraction efficiency are presented in Fig. S7 of SM).

The precision of the extraction method was 5.5, 9.0, 6.5, 5.6, 3.8, 3.4 and the accuracy was 1.0, 2.4, 1.7, 0.8, 1.3, 2.0 for PTE, CAU, PTA, pterosins G, A and B, respectively. The test of the matrix effects demonstrated that plant extracts cause ion enhancement of the 6 analytes. However, the quantification of the analytes was linear after dilution of extracts by 20 or more times (Figs. S8 and S9 of SM). A dilution factor of 25 times was applied resulting in LOQ in the plant samples of 6.3–19.5 μg/g for glycosides and 0.8–2.3 μg/g for pterosins.

3.5. Method application on fern samples

The method was applied for measuring contents of the analytes in the samples of ferns from various regions of the world. In total 18 samples of ferns, 17 of which represented the genus *Pteridium* were extracted in duplicates and analysed in a randomised order. The average coefficients of variation between the measured contents of CAU, PtrA, PTE, PtrG, PTA and PtrB were 0.17, 0.09, 0.01, 0.12, 0.10 and 0.17 respectively. The average measured contents are provided in Table 9.

Illudane glycosides or corresponding pterosins were detected in all samples of *Pteridium* and not detected in the single sample of other fern (No. 3). The concentrations of glycosides in the samples prior to hydrolysis were obtained summing the concentrations of quantified glycosides with concentrations of quantified corresponding pterosins and applying 1:1 M conversion ratios (Table S3 of SM). Relative distributions of glycoside concentrations prior to hydrolysis are presented in Fig. 3.

4. Discussion

The most recently reported LC-MS method measuring PTA and pterosin B in the same instrumental setup used 53% methanol in a 10 min. isocratic elution [30] whereas the new method uses on average 35% of acetonitrile in only 5 min. gradient elution. Two times higher prices of acetonitrile than methanol (European market) make exploitation of the newly developed LC-MS method 1.5 times cheaper. Application of 0.5 mM sodium acetate in the eluent of the previously reported PTA and pterosin B quantification method resulted in precipitation of sodium salts in the mass spectrometer and required regular cleaning of chromatographic column with organic solvents. The method reported in this study does not require additional maintenance neither compromises durability of the equipment.

The LODs of the LC-MS method were 0.22 μg/L for PTA and 0.03 μg/L for pterosin B, whereas the ranges of previously reported methods span from 0.014 to 0.6 μg/L for PTA and 0.008 to 500 μg/L for pterosin B. A single method for quantification of PTE and CAU in GC-MS after conversion of the compounds exists, but the LODs are not specified [29]. The LODs of PTA and pterosin B in the LC-MS method developed in this study do not exceed LODs of the methods applying high-resolution mass spectrometry. However, the presented method applies more affordable single quadruple mass spectrometry and is developed for compound quantification in plants where concentrations of the targeted analytes proved to significantly exceed the LODs. The method reported in this study would quantify but not differentiate between isomers (for example, isoptaquiloside) and would not detect PTA analogues other than PTE and CAU.

The full analytical method allows fast and feasible screening of bracken ferns for wide range of targeted compounds. Its application on samples from six continents of the world revealed high distribution of PTA in the range similar to reported by other studies (0–2,100 μg/g in dry weight). However, the results demonstrated significant concentrations of PTA analogues. PTE was identified in 7 out of 17 samples of bracken. The species in which PTE was originally identified (Australian *P. esculentum*) [27] had the highest proportion of PTE (nearly 80% of the mass of the three glycosides prior to hydrolysis). Nevertheless, quantification in other species of European, African and South-American bracken revealed that PTE comprised up to nearly 20% of the mass of the three glycosides. CAU was identified in 15 samples, in 6 of which its concentrations exceeded PTA. The single PTA neutral bracken sample (European *P. aquilinum* subsp. *Latiusculum*) contained CAU. None of the illudane glycosides were restricted to particular subspecies or origin of bracken. A more extensive empirical study would be required to link biosynthesis of the compounds to factors like genetics or environmental conditions.

The observed relative concentrations of PTA analogues strongly suggest that in most cases the conventional quantification of PTA does not represent the content of the total reactive illudane glycosides. Estimated LogP values and observed order of chromatographic elution demonstrate that the PTA analogues are more soluble than PTA. The illustrated common occurrence together with high solubility suggest significant risk of leaching of the compounds from bracken to soils and water. Previous studies reported PTA in milk of animals grazing cattle (up to 3.1 μg/L) [31,32,51,52] as well as in surface waters and shallow ground waters adjacent to bracken ferns, with peak concentrations

Table 7
Comparison of calibration curves of analytical standards of pterosins stored in different temperatures in time.

Compound	−20 °C			5 °C			Pairwise *t*-test
	Slope	Intercept	r	Slope	Intercept	r	Recovery %
3 weeks							
Pterosin G	343	209	0.996	295	87.6	0.991	86.6 ± 12.6
Pterosin A	698	2,160	0.999	643	1,640	0.994	93.6 ± 3.58
Pterosin B	643	7,050	0.998	647	7,490	0.998	104 ± 3.69
12 weeks							
Pterosin G	2,480	2,820	0.999	2,571	15,300	0.998	112 ± 4.84
Pterosin A	4,390	8,280	0.999	4,280	11,000	0.999	101 ± 1.25
Pterosin B	2,740	29,800	0.998	2,666	21,800	0.999	99.4 ± 2.64

Table 8
Comparison of relative concentrations of the compounds from different extraction methods.

Method		1	2	3	4	5	6	7	8	9	10	11	12
Relative concentration	PTE	85.3	92.6	98.6	93.0	81.8	81.2	87.7	95.4	86.8	99.7	96.9	**100**
(x % of the maximum yield)	CAU	95.0	93.2	94.5	**100**	84.5	73.5	88.5	98.9	90.2	89.9	87.6	91.9
	PTA	85.6	89.2	**100**	71.4	63.3	72.2	64.5	73.0	64.9	84.8	87.5	86.4
	pterosin G	84.4	96.5	99.8	80.7	74.9	84.8	76.8	78.5	76.7	**100**	95.6	98.1
	pterosin A	96.0	95.4	**100**	89.9	167*	83.7	86.5	88.3	87.2	94.8	93.5	93.2
	pterosin B	93.9	**100**	79.5	57.1	52.7	58.6	50.5	46.4	58.1	53.3	67.3	53.2
	Average	*90.1*	**94.5**	**95.4**	*82.0*	*87.4*	*75.7*	*75.8*	*80.1*	*77.3*	*87.1*	*88.1*	*87.1*

* The data point too distant from other data points disregarded as an outlier.

following precipitation of up to 4 μg/L [53–55]. The present study particularly warrants re-evaluation of the total reactive illudane glycosides as environmental contaminants and provides applicable method for preparation of analytical standards and chromatographic separation of analytes. However, the isotope internal standards are not available and the production of analytical standards proved to be complicated due to the hygroscopic nature of the compounds. To foster research of the illudane glycosides, commercial availability of analytical standards and reference materials would be of advantage.

Besides application in research of illudane glycoside fate, toxicity and carcinogenesis, the method could serve studying yet indefinite taxonomy of the genus [1], advancing understanding on its spreading strategies and potential impacts on biodiversity and ecosystem services. Furthermore, the method could serve for enhancing emerging applications of pterosins for pharmaceutical purposes [56–58]. The developed quantification method of the compounds in the samples is directly applicable only for plant material. However, the LC-MS part of the method could as well be adapted and validated for rapid screening of illudane glycosides in non-plant environmental samples. The formerly reported upper concentrations of PTA in water and milk exceed LODs of the LC-MS method presented in this study. However, its application on non-plant environmental samples such as soils, water and food would require combination with sample extraction and preconcentration techniques.

CRediT authorship contribution statement

Vaidotas Kisielius: Conceptualization, Investigation, Writing - original draft. **Dan Nybro Lindqvist:** Conceptualization, Investigation, Supervision. **Mikkel Boas Thygesen:** Investigation, Writing - review & editing. **Michael Rodamer:** Supervision, Resources. **Hans Christian Bruun Hansen:** Supervision, Writing - review & editing. **Lars Holm Rasmussen:** Conceptualization, Supervision, Data curation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 9
Measured content of analytes in diverse fern samples.

Sample No.	Species	Country of origin	Location (WGS84 coordinations)	Measured μg/g (dry weight)					
				CAU	PtrA	PTE	PtrG	PTA	PtrB
1	*P. aquilinum*	Denmark	55.947095, 9.759093	75[b]	133[a]		28[c]	1,370[a]	345[a]
2	*P. aquilinum*	Denmark	55.123898, 14.917845	23[b]	2[b]			5[a]	9[a]
3	*Dryopteris cristata (L) A. Gray*	Denmark	Ordrup Næs peninsula (55.84, 11.37)						
4	*P. aquilinum*	Sweden	56.29849, 13.370333	63[a]	71[a]	177[a]	146[a]	1,530[a]	207[a]
5	*P. aquilinum*	Sweden	57.092993, 14.736379	trace[b]	7[a]		trace[c]	33[a]	12[a]
6	*P. aquilinum* subsp. *latiusculum*	Finland	62.203272, 30.086088	16[b]	24[a]				
7	*P. aquilinum*	Lithuania	55.459371, 26.190076	551[a]	61[a]			6[a]	4[a]
8	*P. aquilinum*	UK	54.50408, −0.83638	38[b]	8[b]			42[a]	35[a]
9	*P. aquilinum*	UK	54.502781, −0.841872	28[b]	21[b]			286[a]	94[a]
10	*P. aquilinum*	UK	54.388075, −0.692965	26[b]	15[b]			213[a]	57[a]
11	*P. aquilinum*	India	Kullu district					trace[b]	5[b]
12	*P. esculentum*	Australia	−27.023612, 153.125651	215[a]	124[a]	2386[a]	856[a]	792[a]	110[a]
13	*P. aquilinum*	Tanzania	Moshi Rural district (−3.293, 37.5)	205[a]	402[a]		53[c]	788[a]	732[a]
14	*P. aquilinum*	Tanzania	Siha district (−3.164, 37.121)				3[c]	34[a]	36[a]
15	*P. arachnoideum*	Brazil	Wanderley municipality		1302[a]		18[c]		26[a]
16	*P. arachnoideum*	Brazil	Wanderley municipality		16[c]		22[c]		88[a]
17	*P. aquilinum*	USA	46.558961, −90.662296	239[a]	814[a]			77[a]	141[a]
18	*P. aquilinum*	USA	46.39491, −89.14131	524[a]	327[a]			57[a]	29[a]

The levels of certainty of determination of the compounds were characterized by the following levels:
[a] Distinctive mass fragments.
[b] Retention time and detection of corresponding pterosin (parent glycoside) in the same sample.
[c] Retention time without emerging distinctive mass fragments due to co-elution of other substances from the plants.

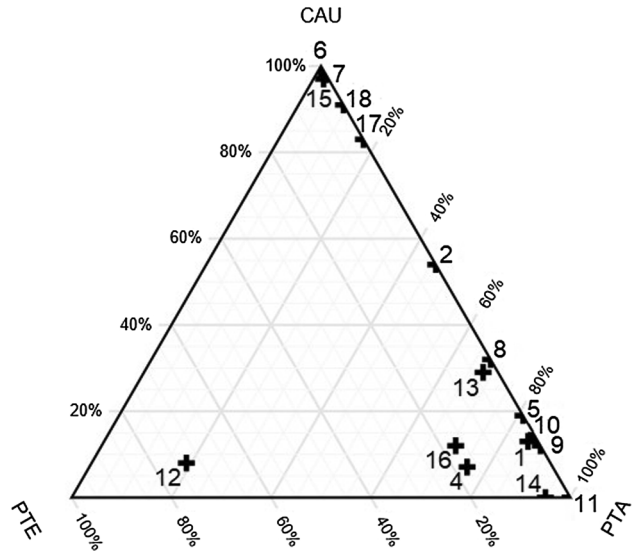


Fig. 3. Ternary distribution of concentrations of glycosides prior to hydrolysis in 17 positive fern samples.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2019.121966.

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